ELSEVIER

UTERINE HORN INSEMINATION OF HEIFERS WITH VERY LOW NUMBERS OF NONFROZEN AND SEXED SPERMATOZOA

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ABSTRACT

Experiments were conducted to determine 1) pregnancy rates of heifers inseminated with very low numbers of spermatozoa under ideal field conditions, and 2) pregnancy rates with low doses of sexed spermatozoa. In Experiment 1, semen from 3 Holstein bulls was extended to 1 x 10^5 or 2.5 x 10^5 sperm/0.1 ml; 2.5 x 10^6 total sperm/0.21 ml were used for the control. Semen was cooled to 5°C, packaged into modified 0.25-ml French straws, and used 26 to 57 h after collection. Spermatozoa were inseminated 24 h after detection of estrus into the uterine horn of Holstein heifers ipsilateral to the ovary with the largest follicle, as determined by ultrasound 12 h after estrus was detected; side of ovulation was verified by detection of a corpus luteum (CL) by ultrasound 7 to 9 d post estrus. Pregnancy was determined 40 to 45 d post estrus. The side of ovulation was determined correctly in 262 of 286 heifers (92%), and pregnancy rates were nearly identical for ipsilateral and contralateral inseminations. Pregnancy rates were 48/118 (41%), 56/111 (50%), and 35/57 (61%) for 1 x 10^5 , 2.5 x 10^5 and 2.5 x 10^6 sperm per insemination ($P<.05$ between 1 x 10^5 and 2.5 x 10^6) . There were no significant differences in pregnancy rates ($P>.05$) among the heifers for the 3 AI technicians or the 3 bulls. In Experiment 2, freshly collected semen was transported from Lancaster, Pennsylvania to Beltsville, Maryland, and sorted into X- and Y-sperm populations based on DNA difference using a flow cytometer/cell sorter over a 6-h period. Sorting rates were about 100 sperm/sec of each sex at ~90% purity. Sorted spermatozoa were shipped ~2600 km by air, and in most cases cooled to 5°C during shipping over 6 h in an Equitainer. Heifers were inseminated with 1 to 2 x 10^5 sorted X- or Y-spermatozoa in 0.1 ml within 9 to 29 h of sorting. The inseminate was either deposited into the uterine horn ipsilateral to the ovary with the largest follicle as determined by ultrasound at the time of insemination, or half of the inseminate was deposited into each uterine horn. None of 10 heifers became pregnant when inseminated with sexed spermatozoa shipped at ambient temperature. Of the 155 heifers inseminated with sexed spermatozoa cooled to 5°C, 15 of 67 females (22.4%) inseminated 9 to 13 h post sorting calved, but only 2 of 78 (2.6%) inseminated at 17 to 29 h calved. Fourteen of the 17 calves born (82%) were of the selected sex.

Key words: sexed spermatozoa, AI, liquid semen, bovine, flow cytometry

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INTRODUCTION

Most research on artificial insemination (AI) of cattle has been done with relatively high numbers of sperm cells to maintain reasonable fertility in the study herds. However, experiments using large numbers of spermatozoa per inseminate are generally much less informative than those with lower numbers because treatment effects that cause lower fertility are masked by the excess number of sperm cells. Research on AI with low numbers of sperm is warranted to develop more informative experiments, obtain more offspring from valuable sires, and determine critical sperm numbers that maintain reasonable fertility for biotechnologies such as sexing spermatozoa (7) where application is restricted by the low rate of sperm sorting with flow cytometry.

Salisbury and VanDemark (19) indicated that fertility in cattle becomes compromised with fewer than 5×10^6 fresh spermatozoa per insemination dose. Jondet (10) reviewed 21 studies, of which only 2 contained treatments using fewer than 10^6 sperm/dose. The lowest insemination dose tested, 0.5×10^6 sperm cells (22), resulted in fertility comparable to that of the control dose, 2.5×10^6 sperm (65 and 68%, respectively, for 49-d nonreturn rates). Since then, considerable advances have been made in semen technology, but very little has been published concerning low sperm numbers in liquid semen except from New Zealand workers. The most relevant of their studies (14) reported nonreturn rates in lactating dairy cows of 65.9 and 60.5% when inseminations were performed late in estrus using 2.5 and 0.5×10^6 sperm/dose, respectively, (n=2041 and 1125). With early to mid-estrus insemination, the nonreturn rates were 62.0 and 52.3% for the same respective doses (n=2461 and 1303). Currently, semen from many bulls in New Zealand is used at an insemination dose of 1×10^6 total sperm cells (K.L. Macmillan, personal communication). This semen is kept at ambient temperature in an extender containing catalase (23). Macmillan and Watson (16) also found that higher pregnancy rates were obtained with post estrus insemination than with mid- to late-estrus insemination when the bulls had average or slightly below average fertility. A recent field trial using liquid semen at 10×10^6 sperm/0.5 ml dose showed that nonreturn rates were 7.5% higher with liquid semen on the day of collection than with frozen semen, but that the higher fertility declined rapidly as liquid semen was used over the next 2 d (11).

Deep uterine insemination of frozen-thawed semen, with half of the semen deposited into each uterine horn, was reported to result in higher pregnancy rates (65%) than when deposited into the uterine body (45%; 21). In another study, Lopez-Gatius and Camon-Urgel (13) deposited semen in the uterine horn ipsilateral to the palpated preovulatory follicle and obtained a 71% pregnancy rate compared with 60% for insemination into the uterine body. McKenna et al. (17), among others, however, showed no advantage to uterine horn insemination.

Sperm sorting using flow cytometry and DNA as a marker for X and Y chromosome-bearing sperm cells was first demonstrated by Johnson et al., who, utilizing surgical insemination, produced a preponderance of offspring of the preselected sex in rabbits (8) and swine (6). The first pregnancies produced from sexed bovine spermatozoa using flow cytometry cell sorting was reported by Cran et al. (2), who used X- and Y-sorted sperm cells for in vitro fertilization followed by embryo transfer. In a field trial with 106 Angus cattle, Y-sorted spermatozoa (3) produced 90% males from resulting embryos which had been frozen and then transferred. For a recent review of sexing spermatozoa by flow cytometry, see Johnson (6).

The present study was undertaken to determine the effectiveness of insemination with low numbers of spermatozoa, 1×10^5 and 2.5×10^5 sperm/dose, and to ascertain if spermatozoa sorted by sex using flow cytometry and then inseminated at these low numbers would result in pregnancies.

MATERIALS AND METHODS

Experiment 1: Liquid Semen

Semen from 3 Holstein bulls of above average fertility based on nonreturn rates of inseminated females was collected using an artificial vagina and processed at Atlantic Breeders Cooperative, Lancaster, Pennsylvania. Neat semen was treated with antibiotics (24) and extended in Cornell Universal Extender (CUE; 14.5 g/l Na citrate-$2H_20$, 2.1 g/l $NaHCO_3$, 0.4 g/l KC1, 3.0 g/l glucose, 9.37 g/l glycine, 0.87 g/l citric acid and 200 ml/l egg yolk) containing 250 μg/ml Gentamycin, 50 μg/ml Tylosin tartrate, 150/300 μg/ml Lincospectin and 5% homologous seminal plasma. Extender was prepared the day prior to use, allowing large particles to settle overnight so that the supernatant could be used. Seminal plasma was obtained by centrifuging previously collected ejaculates twice at 3000 x g and stored in liquid nitrogen. Semen was initially extended 1:10 in CUE containing twice the final antibiotic concentration and cooled to 5°C over 2.5 h. After reaching 5°C, semen was further extended 1:1 with CUE containing 5% final antibiotic concentration. The semen was then further extended serially with CUE containing the final antibiotic concentration and seminal plasma to equal insemination doses of 1×10^5 and 2.5×10^5/0.1 ml spermatozoa or the control insemination dose of 2.5×10^6 sperm/0.21 ml. Sperm concentrations were verified using a hemocytometer. The processed semen was shipped at 5°C to Colorado by overnight commercial courier.

Holstein heifers 13 to 15 mo of age and weighing 350 to 450 kg were injected twice at 12-d intervals with 25 mg $PGF_{2\alpha}$ (Lutalyse®, Upjohn, Kalamazoo, MI) to synchronize estrus. Heifers were tail-painted and observed for estrus at 12-h intervals beginning 36 h after the second injection and continuing through 84 h post injection. Ovaries were examined by ultrasonography (Aloka 500, Corometrics, North Wallingford, CT) with a 5-MHz linear probe 12 h after the first detected estrus to determine which ovary had the largest follicle that would most likely ovulate. Inseminations were performed 24 h after the initial detection of estrus with embryo transfer straw guns and stainless steel-tipped side-opening blue sheaths (IMV, Minneapolis, MN). Semen was deposited between the greater curvature and tip of the uterine horn ipsilateral to the presumed preovulatory follicle 26 to 57 h post collection. The experiment was performed in 5 replicates and was balanced over 3 technicians, each inseminating approximately equal numbers of heifers within each bull-sperm number subgroup. To obtain more information with the lower experimental sperm doses, only half as many inseminations were planned for the controls.

The ovary that actually ovulated was confirmed by ultrasonography of luteal tissue 7 to 9 d post insemination. Pregnancy status was also determined by ultrasonography 40 to 45 d post estrus. Data were analyzed by Chi-square with the Fisher-Yates correction for single degree of freedom comparisons.

Experiment 2: Flow Cytometrically Sorted Sperm

In this preliminary experiment, spermatozoa were sorted based on the DNA content of the X- and Y-chromosome on 9 separate dates, representing 9 different sorting sessions, over a period of 10 mo. Spermatozoa from a single bull were sorted during each session except in Session 2, at which time single ejaculates from 2 bulls were mixed prior to sorting. Semen from 7 Holstein bulls of above average fertility, based on nonreturn rates of inseminated females, and 1 Angus bull of unknown fertility was used in Experiment 2.

Semen was collected via artificial vagina, treated with antibiotics (24), diluted 1:4 in a Hepes-buffered diluent (7.6 gm NaCl, 0.3 0 gm KC1, 2.52 gm fructose, 2.38 gm Hepes, 0. 15 gm $CaCl_2$, 0.10 gm $MgCl_2$, and 1.0 gm bovine serum albumin per liter titrated to pH 7.4; 6) and transported 160 km at ambient temperature to Beltsville, Maryland. Holstein bulls were housed in Lancaster, Pennsylvania, and the Angus bull in Berryville, Virginia. The semen was sorted into X- and Y-bearing sperm populations using

the Beltsville Sperm Sexing Technology. Semen was prepared for sorting using a modification of the standard protocol for viable intact spermatozoa (8). Hoechst 33342 (7.12 μM) was added to a 1-ml aliquot of extended semen containing 15 x 10^6 sperm/ml, followed by incubation for 40 min at 32°C (Johnson and Welch, unpublished data). The sperm cells were sorted based on their DNA content using a modified Epics V/750 series flow cytometer/cell sorter (Coulter Corporation, Miami, FL) as described by Johnson et al. (8) employing an Argon Inova laser operating at 150 mW of power. After incubation, the sperm cells were sorted at room temperature into 0.6-ml BSA-coated presiliconized polypropylene microcentrifuge tubes (Intermountain Scientific, Kaysville, UT) containing 100 μl of TEST-yolk (20%) extender (8) with or without seminal plasma (~5% of the final sorted sperm volume). Sorting rates of ~2 x 10^6 spermatozoa of each sex per 5- to 6-h sorting session were achieved. To validate the proportion of spermatozoa sorted into each population, aliquots of sorted sperm cells were reanalyzed for DNA content (8). Sorted spermatozoa collected in microcentrifuge tubes were concentrated by centrifugation (300 x g for 4 min) to 1.6 x 10^6 sperm/ml and shipped by commercial air to Colorado (2600 km) at ambient temperature or cooled to 5°C en route in an Equitainer (4). Cooled, sorted semen was packaged into cooled 0.25-ml French straws in a 5°C cold room in Colorado and taken to the breeding area in insulated containers.

As described in Experiment 1, estrus-synchronized females were inseminated by 2 technicians on 9 separate days with flow-sorted spermatozoa (1 to 2 x 10^5 in 0.1 ml) within 9 to 29 h of the sorting session. Approximately equal numbers of females were inseminated with either X or Y chromosome-bearing spermatozoa. On Dates 1 and 2, ten heifers and nonlactating cows were inseminated, as described in Experiment 1, with sorted spermatozoa shipped and maintained at ambient temperature. On Date 2, nine females, and on subsequent dates all heifers were inseminated with sorted spermatozoa cooled to 5°C. Inseminations were performed as described in Experiment 1 except 0.05 ml sorted sperm cells were deposited into each uterine horn on Dates 4, 5 and 6 without using ultrasonography to predict the side of ovulation.

Pregnancy was determined at 4 wk post estrus and confirmed at 2 mo post estrus by ultra-sonic visualization of a viable fetus and by calving. Data were analyzed by Chi-square with the Fisher-Yates correction.

RESULTS

Experiment 1

Pregnancy rates are shown in Table 1. Side of ovulation was predicted correctly in 262 of 286 heifers (92%). However, there was no difference ($P>0.1$) in pregnancy rates between inseminations ipsilateral and contralateral to the side of ovulation. Pregnancy rates differed ($P<.05$) between the 2 doses, 1 x 10^5 and 2.5 x 10^6 sperm/inseminate (41 vs 61%), but were not significantly ($P>0.1$) affected by technician or bull ($P>0.1$).

Experiment 2

A total of 165 females was inseminated with spermatozoa sorted according to DNA content. There was no significant effect of seminal plasma, so data for this effect were pooled. No pregnancies were obtained from sorted spermatozoa maintained at ambient temperature during transit (n=10), and data for these were not considered further. Of the sorted spermatozoa cooled to 5°C during transit, 22 of 117 females (19%) were pregnant at 4 wk post estrus (Table 2); the 38 heifers inseminated at Session 5 were not tested for pregnancy at 4 wk. By 2 mo post estrus, only 17 (11%) of 155 females inseminated with cooled, sorted spermatozoa remained pregnant, representing an embryo/fetus loss of 23%. There was no fetal loss between 2 mo of gestation and term. In 74 of the 155 females, 0.1 ml of semen was deposited

deep into the uterine horn ipsilateral to the presumed preovulatory follicle, and 81 females were inseminated with 0.05 ml extended semen deep into both uterine horns. The resulting 2-mo pregnancy rates were 22 and 1%, respectively. However, the semen deposition procedure was confounded with elapsed time between sperm sorting and insemination (Table 4). Pregnancy rates of females at 2 mo post estrus ranged from 0 for Bulls 1, 5, 6, and 7 to 35% for Bull 4 (Table 2). Sperm motility was not assessed rigorously prior to insemination because few spermatozoa were available. However, progressive spermatozol motility, as evaluated subjectively, constituted less than 20% of the cells at the time of insemination, particularly for the longer periods post sorting.

Table 1. Pregnancy rates (main effects means) with nonfrozen semen (Experiment 1).

Factor	Side[a]	n	% Pregnant
Sperm/inseminate			
1×10^5	Ipsilateral	104	41[b]
	Contralateral	14	36
2.5×10^5	Ipsilateral	103	50[b,c]
	Contralateral	8	50
2.5×10^6 (control)	Ipsilateral	55	62[c]
	Contralateral	2	50
Technician			
A		93	48
B		99	52
C		94	46
Bull			
1		93	51
2		97	51
3		96	45

[a] Side of insemination relative to ovulation.
[b,c] Values without a common superscript differ ($P<.05$).

A similar pregnancy rate resulted from insemination of Y-bearing spermatozoa compared with that of X-bearing spermatozoa (Table 3); three presumptive female pregnancies and two presumptive male pregnancies were lost between 1 and 2 mo of gestation.

For sperm cooled to 5°C, time of insemination relative to the end of the sorting session affected pregnancy rates (Table 4). Of 67 females inseminated from 9 to 13 h post sorting, 17 (25%) were pregnant at 1 mo post estrus, and 15 (22%) remained pregnant at 2 mo of gestation. For 78 females inseminated 17 to 29 h post sorting, there were 5 (6%) pregnancies at 1 mo (pregnancy diagnosis was not performed on 38 females at Day 28) and 2 (3%) pregnancies by 2 mo of gestation. Time of insemination ranged from 8 to 24 h post sorting for the 10 inseminations (resulting in no pregnancies) with sperm maintained at ambient temperature. Caution must be exercised in interpreting data in Table 4 because time of insemination is confounded with bull effects.

At parturition, all calves appeared phenotypically normal, although 3 born to smaller heifers died due to dystocia; 14 of 17 calves were of the predicted sex, which is significantly different ($P<0.02$) from a

Table 2. Pregnancy rates of females inseminated with sexed spermatozoa cooled to 5°C as presented by bull and insemination session (Experiment 2).

AI session	Bull	Number of females inseminated	Number (%) pregnant 4 weeks	2 months
1	1	4	0	—
2	2,3	15	3 (20)	3 (20)
3	4	20	11 (55)	9 (45)
4	5	17	1 (6)	1 (6)
5	6	38	ND[a]	0
6	7	16	2 (13)	0
7	1	16	0	—
8	4	14	4 (29)	3 (21)
9	8	15	1 (7)	1 (7)
Total		155	22 (19)	17 (11)[b]

[a] Pregnancy determinations were not performed.
[b] All of these pregnancies continued to term.

Table 3. Number of female and male pregnancies by insemination date (Experiment 2).

	Total number of inseminations		Number pregnant 1 month post estrus		2 months post estrus	
Date	X-sort[a]	Y-sort[a]	X[a]	Y[a]	Xa	Y[a]
2	8	7	2	1	2	1
3	12	8	5	6	4	5
4	8	9	1	0	1	0
6	8	8	1	1	0	0
8	8	6	1	3	0	3
9	8	7	1	0	1	0
Total[b]	52	45	11	11	8	9

[a] Numbers corrected for sex of calf in the 3 cases in which expected sex did not match sex of calf.
[b] Totals do not include insemination sessions at which no pregnancies were obtained, when there were 29 X and 29 Y inseminations.

theoretical 50:50 ratio. Five of 5 calves predicted as female (X-bearing sort) were correct, and 9 of 12 predicted as male (Y-bearing sort) were male; 3 were female. The overall accuracy of sexing was 82%; the predicted accuracy derived from resorting a small volume of sorted, sonicated sperm from each session averaged 90% (Table 5).

Table 4. Pregnancy rates using flow sorted sexed sperm cooled to 5°C relative to the time of insemination (Experiment 2).

Time[a] (hours)	Number inseminated	Number (%) pregnant	
		1 month	2 months
9	21	3 (14.3)	3 (14.3)
10	5	4 (80.0)	4 (80.0)
11	11	6 (54.5)	5 (45.5)
12	14	4 (28.6)	3 (21.4)
13	16	0	0
17	4	1 (25.0)	0
18	26	2 (7.7)	1 (3.8)
19	1	0	0
20	3	0	0
22	38	ND	0
26	2	1 (50.0)	1 (50.0)
29	4	1 (25.0)	0

[a] Time from the end of the sperm sorting session to insemination.

Table 5. Proportion of X or Y spermatozoa in the population of sperm sorted for X or Y (Experiment 2).[a]

AI session	% X-chromosome bearing	% Y-chromosome bearing
1	94	91
2	82	90
3	84	90
4	88	88
5	95	95
6	93	94
7	94	96
8	87	88
9	88	86
Mean	89.4	90.8

[a] A small volume of sorted sperm from each session was reanalyzed for DNA content to ascertain the proportions of X or Y sperm in each population (8).

DISCUSSION

The side of ovulation was predicted correctly in 92% of heifers, but pregnancy rates did not differ between contralateral and ipsilateral inseminations (Table 1). With such small numbers of contralateral inseminations, no definitive conclusions could be made. However, Lopez-Gatius and Camon-Urgel (I 3) and Lopez-Gatius (I 2) reported higher conception rates in post partum cows when semen was deposited ipsilateral to the impending ovulation.

The main objective of Experiment 1 was to determine pregnancy rates using sperm numbers just below the threshold for normal fertility but under ideal field conditions. To maximize fertility, we incorporated the following concepts: liquid instead of frozen semen, heifers instead of cows, uterine horn instead of uterine body insemination (17,21), addition of 5% homologous seminal plasma (1,5), use of a small inseminate volume (0.1 ml) to minimize effects of dilution (9), prostaglandin synchronization instead of spontaneous estrus (15), insemination ipsilateral to predicted ovulation (13), use of atraumatic, side-opening sheaths instead of standard AI sheaths, inseminating 24 instead of 12 h after detected estrus (14), use of bulls with higher than average fertility, and use of well-trained technicians. Note that some of these concepts, e.g., addition of seminal plasma and late insemination, may be inappropriate under more conventional conditions. We applied these concepts to all inseminations.

A limitation of the study is the relatively low numbers of inseminations per sperm dose. However, treatments were balanced over 3 bulls and 3 technicians; pregnancy was determined using ultrasound at 6 wk of gestation rather than from nonreturn data; sperm numbers actually delivered from the straws were confirmed using a hemacytometer; and the work was replicated with 3 ejaculates per bull on separate occasions. Another limitation is that bulls were located 2600 km from the heifers, and timing and temperature conditions of semen shipment were not under our control.

Despite these limitations, we clearly demonstrated that pregnancy rates of 40 to 50% are attainable in Holstein heifers using 1 to 2.5 x 10^5 total sperm cells per inseminate. Pregnancy rates with these low sperm numbers were clearly below those of the controls (2.5 x 10^6 sperm/dose), so these very low doses will usually be inappropriate for commercial purposes. However, our work has been followed by logistically easier experiments with low doses of frozen semen (20) that show considerable promise for commercial applications.

The current study resulted in the first offspring produced in any species by nonsurgical artificial insemination of semen sexed by proven methods. The experiments are preliminary in nature, and various permutations of procedures often were done in parallel, that is before pregnancy results from previous procedures were known. The methods were also logistically demanding since the bulls, flow cytometer/cell sorter facilities, and inseminated heifers were quite distant from each other.

Two factors appeared to affect pregnancy rates: bulls and time between sorting and insemination. Semen from Bull 4 (Table 2) resulted in 12 term pregnancies from 34 inseminations (35%), a higher number ($P<.05$) than that from most of the other bulls. There also appears to be a marked decline in pregnancy rates by 17 h after the end of sorting compared with 13 h or less. The 13 h and earlier time periods represent inseminations performed within a few hours of the arrival of semen at the Colorado Laboratory (generally 10 PM to 2 AM inseminations), whereas those 17 h and later represent inseminations done the following day (usually 6 AM to 6 PM). A major reason for inseminating so many heifers 17 to 22 h post sorting was that 2 of 6 heifers were pregnant at 4 wk from later inseminations in the early replicates.

Five of 22 pregnancies (23%) were lost between 1 and 2 mo of gestation. Three of these were from the late inseminations, which is greater, 3/5 (60%), than that of early inseminations, 2/17 (12%; $P<0.1$). Greater numbers of pregnancies from sexed semen need to be analyzed to determine if embryonic death increased with flow-sorted bovine spermatozoa. McNutt and Johnson (18) found an increase in embryonic deaths resulting from the use of flow-sorted rabbit spermatozoa; however, they had also used a higher laser power and a higher concentration of dye.

Five of five pregnancies with spermatozoa sorted as X-chromosome-bearing resulted in females, whereas 9 of 12 sorted as Y-chromosome-bearing spermatozoa resulted in males (2 calves from 1 sorting session were of the wrong sex). Data are too few to determine if Y-sorting was less accurate than X-

sorting. The 82% accuracy of the sex of the calves born is not significantly different from the 90% expected (Table 5).

Experiment 2 is a combination of successes and failures (Table 2). This work needs to be repeated under more controlled conditions that are logistically less demanding. For commercial application, the sorting speed needs to be increased considerably without increasing damage to sperm cells while maintaining reasonable accuracy, preferably 90% or more. Furthermore, for widespread application, fertility must be maintained without increasing embryo mortality. Although cryopreservation is not essential for all applications utilizing sexed spermatozoa, cryopreservation of sexed spermatozoa would greatly facilitate application.

REFERENCES

1. Ashworth PJC, Harrison RAP, Miller NGA, Plummer JM, Watson PF. Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. Reprod Fertil Dev 1994;6:173-180.
2. Cran DG, Johnson LA, Miller NGA, Cochrane D, Polge C. Production of bovine calves following separation of X- and Y-chromosome-bearing sperm and in vitro fertilization. Vet Rec 1993;132:40-41.
3. Cran DG, Johnson LA, Polge C. Sex preselection in cattle: a field trial. Vet Rec 1995;136:495-496.
4. Douglas-Hamilton DH, Osol R, Osol G, Driscoll D, Noble H. A field study of the fertility of transported equine semen. Theriogenology 1984;22:291-304.
5. Henault MA, Killian GJ, Kavanaugh JF, Griel LC Jr. Effect of accessory sex gland fluid from bulls of differing fertilities on the ability of cauda epididymal sperm to penetrate zona-free bovine oocytes. Biol Reprod 1995;51:390-397.
6. Johnson LA. Sex preselection by flow cytometry. Separation of X and Y chromosome bearing sperm based on DNA difference: A review. Reprod Fertil Dev 1995;7:893-903
7. Johnson LA, Cran DG, Polge C. Recent advances in sex preselection of cattle: flow cytometric sorting of X- and Y-chromosome bearing sperm based on DNA to produce progeny. Theriogenology 1994;41:51-56.
8. Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 1989;41:199-203.
9. Jondet R. Influence de la reduction du volume de la dose de sperme congele et de sa teneur en spermatozoides sur la fecondation des vaches inseminees artificiellement. VI Int Congr Anim Reprod Artif Insem 1968;11: 1061-1063.
10. Jondet R. Transport des spermatozoides chez la vache et fertilite apres insemination artificielle avec des nombres reduits de spermatozoides. INSERM 1973;26:357-388.
11. Kaproth M. Experiences with liquid semen -- an early field trial. Proc Am Embryo Transfer Assoc 1993;28-38.
12. Lopez-Gatius F. Site of gestation in dairy heifers affects subsequent sperm transport and pregnancy rates after deep insemination into one uterine horn. Theriogenology 1996;45:417-425.
13. Lopez-Gatius F, Camon-Urgel J. Increase of pregnancy rate in dairy cattle after preovulatory follicle palpation and deep cornual insemination. Theriogenology 1988;29:1099-1103.
14. Macmillan KL, Curnow RJ. Factors influencing A.B. conception rates. XI. Variation in sire fertility. NZ J Exp Ag 1977;5:279-285.
15. Macmillan KL, Day AM. Prostaglandin F-2-alpha -- a fertility drug in dairy cattle? Theriogenology 1982;18:245-253.
16. Macmillan KL, Watson JD. Factors influencing A.B. conception rates. VII. Interactions involving semen fertility levels, sperm dose rate, and technician ability. NZ J Exp Ag 1977;5:11-14.
17. McKenna T, Lenz RW, Fenton SE, Ax RL. Nonreturn rates of dairy cattle following uterine body or cornual insemination. J Dairy Sci 1990;73:1779-1783.

18. McNutt TL, Johnson LA. Flow cytometric sorting of sperm: Influence on fertilization and embryo/fetal development in the rabbit. Mol Reprod Dev 1996;43:261-267.
19. Salisbury GW, VanDemark NL. Physiology of Reproduction and Artificial Insemination of Cattle. San Francisco: Freeman and Company, 1961.
20. Seidel GE Jr, Allen CH, Brink Z, Holland MD, Cattell MB. Insemination of heifers with very low numbers of frozen spermatozoa. J Anim Sci 1996;74(Suppl. 1):235 abstr.
21. Senger PJ, Becker WC, Davidge ST, Hillers JK, Reeves JJ. Influence of cornual insemination on conception in dairy cattle. J Anim Sci 1988;66:3010-3016.
22. Shannon P. The effect of egg yolk level and dose rate on conception rate of semen diluted in caprogen. VII Int Congr Anim Reprod Artif Insem 1972; 11: 1441-1442.
23. Shannon P, Vishwanath R. The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. Anim Reprod Sci 1995;39: 1 -10.
24. Shin S. New antibiotic combination for controlling ureaplasma, mycoplasmas, Haemophilus sommus, Campylobacter fetus, and leptospiras in frozen bovine semen. Proc XI NAAB Tech Conf 1988;33-38.